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Altered expression of annexin II in human B-cell lymphoma cell lines

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Abstract

Annexin II is a growth-regulated gene, whose expression is significantly increased in various human cancers. We examined annexin II expression in 11 human B-cell lymphoma cell lines and in normal B-cells. Wide variation was observed in the levels of annexin II in these cell lines. Annexin II overexpression was observed in 5 cell lines, while significantly reduced expression was observed in Raji, OMA-BL-1 and REH cell lines. Analysis of the annexin II gene, mRNA and protein in Raji and OMA-BL-1 cell lines indicated that annexin II gene was unaltered and that a low level of annexin II transcripts are produced in these cells. Down-regulation of annexin II expression was at the transcriptional level, and no reexpression of annexin II was observed after treatment of cells with demethylating agents. Thus methylation of the annexin II gene does not appear to be responsible for annexin II down-regulation. A slow migrating altered form of annexin II was detected in Raji and OMA-BL-1 cells, which was detected with the anti-chicken annexin II antiserum, but not with the anti-human annexin II antiserum. The slow migrating annexin II species was found to be sensitive to dephosphorylation by calf intestinal alkaline phosphatase, resulting in reduction of the size of the protein on SDS-polyacrylamide gels. The phosphorylated annexin II was also observed in nuclear extracts of human K562 and HeLa cells. Thus, Raji and OMA-BL-1 cells exclusively produce a phosphorylated form of annexin II, and phosphorylated annexin II may be important for cell survival and proliferation.

Keywords: Annexin II; Phosphorylation; Cell proliferation; B-cell lymphoma; DNA replication

1. Introduction

Annexin II (also called calpactin I, lipocortin II, p 36) is a major cellular substrate for phosphorylation by various receptor- and non-receptor protein kinases (reviewed in [1]). The mechanism of transduction of mitogenic signals downstream of the initial phosphorylation event remains to be elucidated. Annexin II is present in cells as a monomer, and in association with calpactin I light chain (p11), a heterotetramer is formed which is found associated with the plasma membrane [2]. The monomer annexin II is seen in the nucleus [3,4], and extracellular occurrence of annexin II is also reported [5]. Based on its distribution in cells, various physiological functions have been assigned to various forms of annexin II, such as mitogenic signal transduction [6], DNA synthesis and cell proliferation [7], membrane fusion during exocytosis and endocytosis [8,9], cation-dependent adhesion of tumor cells to endothelium [5], and as a receptor for tissue plasminogen activator [10,11].

We have previously reported an interaction of annexin II with DNA polymerase α in in vitro DNA replication [7]. Addition of antisense annexin II oligodeoxynucleotides to growing HeLa cells reduces ongoing DNA synthesis and retards cell cycle progression [12]. Immunodepletion of annexin II from *Xenopus* egg extracts results in inability of the extracts to support DNA synthesis, while addition of purified annexin II to immunodepleted extracts restores the ability to synthesize DNA [13]. Annexin II levels are increased in a variety of cancers [14–18], further supporting a role of annexin II in cell proliferation. In this study, we examined annexin II expression in human B-cell lymphoma cell lines. We found a wide variation in annexin II expression in these cell lines. Two cell lines that have significantly reduced levels of annexin II were examined further. The data reported in this paper demonstrates that these two cell lines produce the phosphorylated form of annexin II exclusively. Thus, phosphorylated annexin II may be necessary for cell proliferation.

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2. Materials and methods

2.1. Cell culture, drug treatment and reagents

The human B-cell lymphoma cell lines used in this study are: progenitor B-cell lines (Nalm-6, REH, HPB-Null, PBE-I), B-lymphoblast cell lines (WI-L2, TK-6, DW-10) and Burkitt's lymphoma cell lines (Raji, Ramos, OMA-BL-1, Namalwa). Human adenoids were used as a source of normal B-cells, and by cell sorting we determined that adenoids contained > 80% B-cells. HeLa and K562 cells were used as positive controls in all experiments. Cells were grown in a growth medium consisting of Eagle's minimum essential medium (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). All cell lines were determined to be Mycoplasma-negative by a PCR-based Mycoplasma detection assay. 5-aza-2′-deoxycytidine (deoxyC, Sigma Chemical Co., St. Louis, MO) was freshly prepared in distilled water, and cells were treated as indicated in figure legends. Genomic DNA, total cellular RNA and protein were extracted from cells using previously published procedures [16,17]. Dephosphorylation of extracts was performed by incubation of 20 μg of extract with 2 units of calf intestinal alkaline phosphatase (Gibco-BRL, Grand Island, NY) at 37°C for 30 min in 50 mM Tris-HCl, 0.1 mM EDTA (pH 8.5). Dephosphorylated extracts were then subjected to SDS-PAGE and immunoblot analysis.

2.2. Antibodies

Polyclonal rabbit anti-human annexin II antisera (α774) was a gift from Dr. Blake Pepinsky (Biogen, Boston, MA). Polyclonal rabbit anti-chicken annexin II antiserum was generously provided by Dr. Tony Hunter, Salk Institute, La Jolla, CA. Polyclonal anti-human phosphoglycerate kinase antisera was prepared as described previously [4].

2.3. Gel electrophoresis and immunoblotting

Proteins were resolved on 4–15% polyacrylamide gels under denaturing conditions and electrotransferred onto Immobilon-P (Millipore Corp, Boston, MA) transfer membranes as described previously [7]. The membranes were subjected to immunoblotting as described previously [7]. The blots were exposed to a Kodak X-Omat AR film with a Cronex-plus intensifying screen at −80°C. For quantitation of the immunoreactive band, the blot was scanned on a Betascope 603 (Betagen, Waltham, MA) radioanalytical imager.

2.4. Analysis of steady-state annexin II message levels

Steady-state mRNA levels of annexin II was examined by ribonuclease protection assay and Northern blotting. Total RNA was isolated from cells and 5 μg of total RNA was used in the ribonuclease protection assay. A 463-bp *Eco*RI/*Pst*I fragment of annexin II cDNA (corresponding to nucleotides 9-471 from pGAF5 [15]) was cloned into pGEM4Z vector (Promega, Madison, WI) and was used to transcribe a radiolabeled probe. Ribonuclease protection assays were performed using the RPAII kit (Ambion, Austin, TX) as directed. Briefly, each reaction contained 5 μg of RNA and 2×10^5 cpm of ^{32}P-labeled probe. Control reactions were performed simultaneously using 5 μg of yeast tRNA. Hybridizations were carried out for 16 h at 42°C. Ribonuclease digestions were then performed using a mixture of RNase A and T_1 for 1 h at 37°C. Reaction products were separated on a 6% polyacrylamide-urea gel. The dried gel was scanned on a Betascope 603 (Betagen, Waltham, MA) and radioactivity in each band was quantitated. Twenty μg of total RNA was used in Northern blot experiments. Annexin II probe was prepared from the vector pGAF5 [15] by the random primer method of labeling to a specific activity greater than 1×10^8 cpm/μg. Hybridization of the probe to the membrane was carried out at 42°C overnight. Quantitation of blots was done by scanning the blots on a Betascope 603 radioanalytical imager (Betagen) and by cutting out the respective area of the blot and determining radioactivity by liquid scintillation counting. Expression of the message for glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a control in both Northern blots and RPA to normalize data for annexin II levels to account for variation in loading the gel.

2.5. Southern blot analysis

Southern blot analysis [19] of genomic DNA was performed using annexin II cDNA from pGAF5-1 (1.0 kb), radiolabeled as described under Northern blotting, as a probe.

3. Results

3.1. Expression of annexin II in human B-cell lymphoma cell lines

Annexin II protein levels in various B-cell lymphoma cells and other control cells were measured by immunoblot analyses. A typical autoradiogram is shown in Fig. 1. Extracts made from adenoid cells (containing > 80% B-cells) were used as control normal cells, and HeLa cell extracts were used as positive controls. The data from Fig. 1 indicate a wide variation in annexin II expression in the cell lines. Normal B-cells (lane 14) contain detectable levels of annexin II. B-lymphoblast cell lines TK-6 and WI-L2, and a Burkitt's lymphoma cell line Ramos expressed annexin II at the level of normal B-cells. Other cell lines such as HPB-Null, DW-10, Nalm-6, Namalwa and

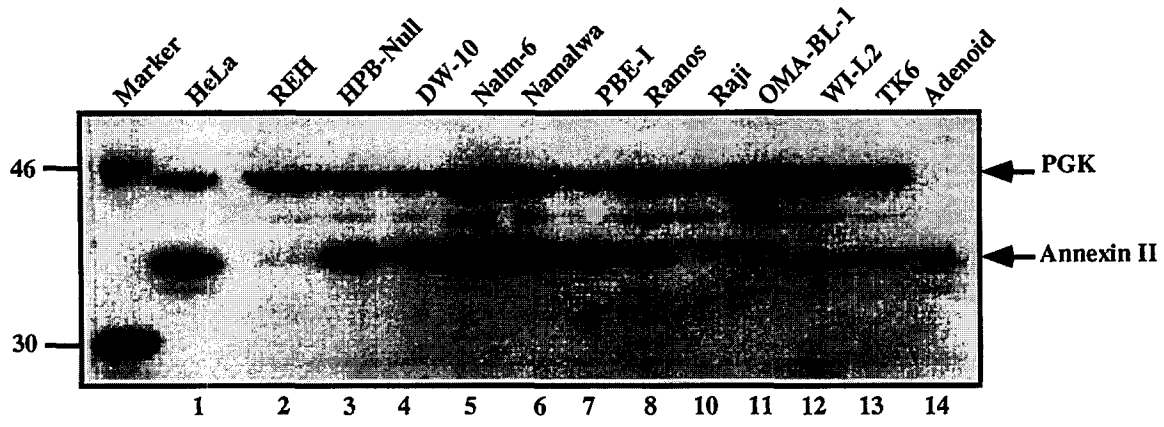

Fig. 1. Expression of annexin II in human B-cell lymphoma cell lines. Whole cell extracts were prepared from adenoids, HeLa and various B-cell lymphoma cell lines. Extracts equivalent to 20 μg of protein were subjected to SDS-gel electrophoresis and immunoblotting. Proteins on the blot were reacted with a 1:2000 dilution of polyclonal α774 antiserum and 1:500 dilution of anti-human PGK antiserum (Rabbit 3.5), and the reactivity was assessed using ^{125}I-labeled protein A. The blot was exposed to an X-ray film for 66 h at room temperature. Radioactivity in the immunoreactive bands was quantitated by scanning the blot on a Betascope 603 radioanalytical imager. Positions of the molecular weight markers, PGK and annexin II are indicated.

PBE-1, had significant overexpression of annexin II. REH and two Burkitt's lymphoma cell lines, Raji and OMA-BL-1 showed no annexin II protein. Since all cultured cells analyzed thus far displayed annexin II expression, we analyzed annexin II expression in these cell lines by repeating immunoblots many times and by radioimmunoassay (data not shown), and confirmed lack of annexin II expression in these cell lines. However, the expression of 3-phosphoglycerate kinase (Fig. 1), proliferating cell nuclear antigen and the 70 and 90 kDa subunits of Ku autoantigen, were unaffected in these cell lines, indicating a specific lack of expression of annexin II.

3.2. Lack of annexin II in Raji and OMA-BL-1 cell lines is transcriptionally regulated

We further examined annexin II expression in Raji and OMA-BL-1 cells by using immunoblot, Northern blot,

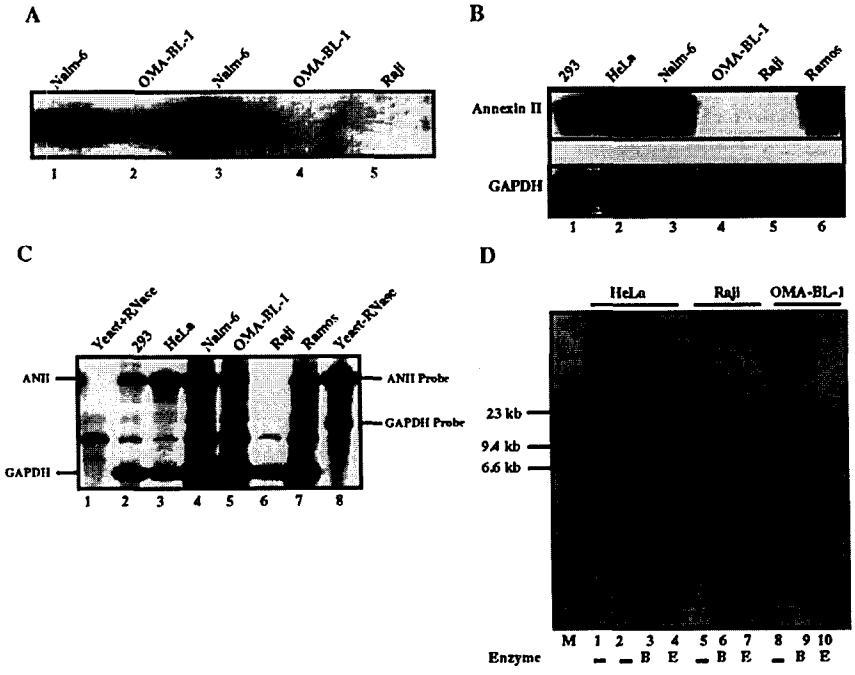

Fig. 2. Analysis of annexin II protein, RNA and gene in Raji and OMA-BL-1 cell lines. Panel A shows a Western blot analysis of annexin II protein levels in different preparations of Nalm-6, OMA-BL-1 and Raji cell extracts. Western blot analysis was done as described in Fig. 1. Panel B shows Northern blot analysis of annexin II mRNA expression in the control 293 and HeLa cells, annexin II-positive Nalm-6 and Ramos cell lines and annexin II negative OMA-BL-1 and Raji cell lines. Panel C shows the results of ribonuclease protection analysis. Positions of the annexin II (470 bp) and GAPDH (395 bp) probes and the protected annexin II (463 bp) and GAPDH (316 bp) fragments are indicated. Panel D shows Southern blot analysis of HeLa, Raji and OMA-BL-1 cells. Genomic DNA was digested with *Bam*HI (B) and *Eco*RI (E) as indicated and Southern blot analysis was performed as described in Section 2. Positions of the molecular weight markers are indicated.

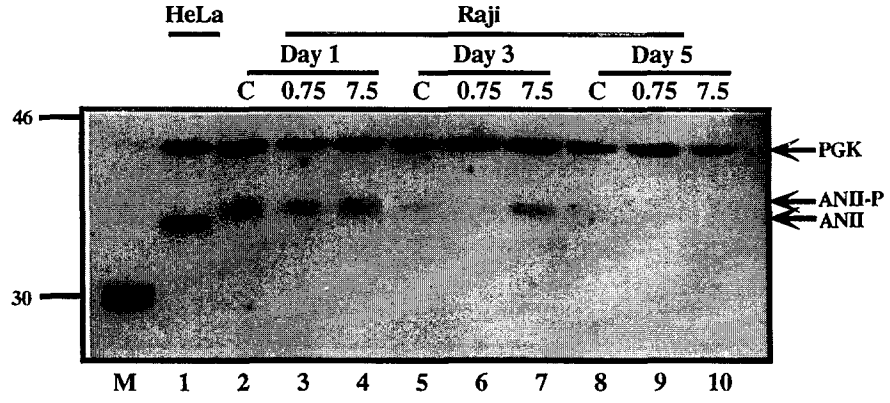

Fig. 3. Methylase inhibitor 5-aza-2′-deoxycytidine does not cause reexpression of annexin II in Raji cells. Raji cells were cultured in the absence (C, lanes 2,5,8) or presence of 0.75 μM (lanes 3,6,9) and 7.5 μM (lanes 4,7,10) of 5-aza-2′-deoxycytidine for 1–5 days as indicated. Protein extracts made from cells were subjected to Western blot analysis as in Fig. 1, with the exception of using anti-chicken annexin II polyclonal antiserum (1:1000 dilution) as the primary antibody. The blot was exposed to X-ray film for 24 h at −80°C.

ribonuclease protection and Southern blot analyses (Fig. 2). Repeated immunoblot analyses indicated lack of annexin II protein (panel A). Northern analysis of total cellular mRNA indicated specific lack of expression or down-regulation of annexin II in Raji and OMA-BL-1 cells (panel B). When Northern blot analysis was repeated many times, in some blots a faint hybridization was visible in Raji and OMA-BL-1 cells. Therefore, we used a more sensitive ribonuclease protection assay to measure annexin II mRNA levels (panel C). In repeated ribonuclease protection analyses, we found that Raji and OMA-BL-1 cells have greatly reduced annexin II mRNA. Compared to another human Burkitt's lymphoma cell line, Ramos, the Raji and OMA-BL-1 cells had a 40- to 50-fold reduction in annexin II mRNA levels (normalized to levels of GAPDH). There was no alteration in the annexin II genomic DNA as seen by no change in the hybridization pattern after cleavage by restriction enzymes (panel D). Therefore, we conclude that the down-regulation of annexin II expression in Raji and OMA-BL-1 cells is at the transcriptional level.

3.3. Down-regulation of annexin II expression in Raji cells is not due to gene methylation

Human annexin II gene has been isolated and an examination of upstream sequences reveal a highly GC rich region (72% GC), with a high frequency of C_pG dinucleotide [20]. Methylation of the C_pG dinucleotide has been shown to directly inhibit transcription or stabilize structural changes in chromatin that prevent transcription [21,22]. We therefore used the nucleotide analogue, deoxyC, to inhibit DNA methylation in Raji cells and measure reexpression of annexin II mRNA and protein. Exposure of Raji cells to either low (0.75 μM) or high (7.5 μM) deoxyC did not result in reexpression of annexin II mRNA (data not shown). When immunoblot analysis was performed using the polyclonal α774 antisera, we did not observe an immunoreactive protein band. However, when the polyclonal anti-chicken annexin II antisera was used, we observed a slower migrating immunoreactive protein band (Fig. 3). The slow migrating protein is a modified form of annexin II, since the anti-chicken annexin II

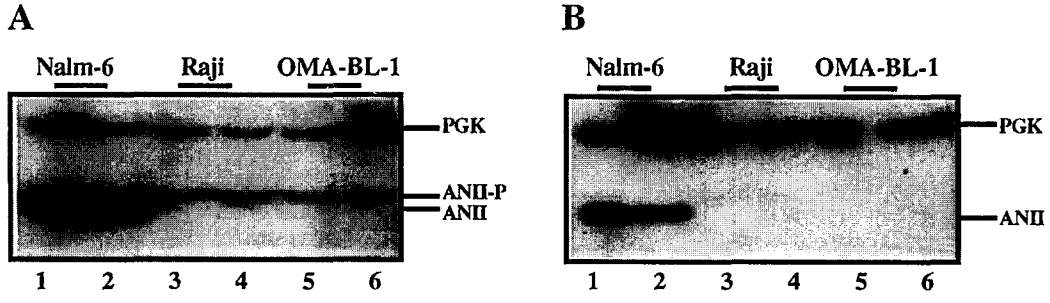

Fig. 4. Raji and OMA-BL-1 cells express an altered form of annexin II. Whole cell extracts (20 μg protein) from Raji, OMA-BL-1 and Nalm-6 cells were subjected to SDS-PAGE and Western blot analysis as described in Fig. 1. In panel A, the blot was developed with polyclonal anti-chicken annexin II antisera (1:1000 dilution), and in panel B, α774 antisera (1:2500 dilution) was used. In both panels, anti-PGK was used at 1:1000 dilution. The blots were exposed to X-ray film at −80°C for 48 h.

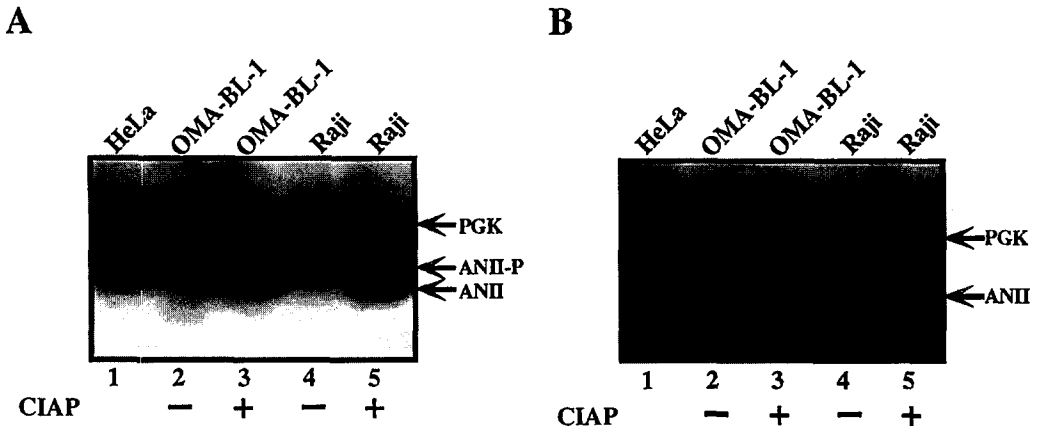

Fig. 5. Phosphorylated annexin II is expressed in Raji and OMA-BL-1 cells. Whole cell extracts from Raji and OMA-BL-1 cells were left untreated (lanes 2 and 4) or treated with calf intestinal alkaline phosphatase (lanes 3 and 5). SDS-PAGE and immunoblot analysis of untreated and dephosphorylated extracts were performed as described in Fig. 1 with anti-chicken annexin II antiserum (panel A) and α774 antiserum (panel B), along with anti-PGK antiserum (in both panels). HeLa cell extracts were included to indicate positions of annexin II and PGK migration.

antiserum does not cross-react with other annexins or other cellular proteins in crude cell homogenates from several cell lines examined by us thus far [4,7,12,16].

3.4. Expression of phosphorylated annexin II is seen in Raji and OMA-BL-1 cells

We further investigated the nature of the slower migrating annexin II protein in Raji and OMA-BL-1 cells. Fig. 4 illustrates results of immunoblot analyses of Nalm-6, Raji and OMA-BL-1 cell extracts with α774 (panel A) or anti-chicken annexin II (panel B) antisera. Annexin II from Nalm-6 cells is immunoreactive with both antisera. However, the slower migrating annexin II protein seen in Raji and OMA-BL-1 cells is immunoreactive only with the anti-chicken antisera. Annexin II is a major cellular substrate for protein-tyrosine kinases and the physiological role of phosphorylated annexin II is unknown. We investigated the possibility that the slower migrating annexin II is the phosphorylated form. Raji and OMA-BL-1 cell extracts were dephosphorylated with calf intestinal alkaline phosphatase, and immunoblot analysis was conducted on untreated and dephosphorylated extracts (Fig. 5). The data show a change in the mobility of the slower migrating annexin II protein upon dephosphorylation in both Raji and OMA-BL-1 extracts, and the dephosphorylated annexin II migrates to the same position as annexin II from Nalm-6 and HeLa cells. Thus, Raji and OMA-BL-1 cells produce phosphorylated annexin II exclusively, and at a significantly reduced amount compared to other cell lines. The dephosphorylated form of annexin II fails to be recognized by the α774 antiserum. We further investigated if the phosphorylated annexin II is associated with nuclei in other cell lines also. Previously, we have identified a slower migrating annexin II species in HeLa nuclear extracts [4]. We prepared nuclear and cytosolic extracts from a human erythroleukemia cell line K562, and subjected these extracts to dephosphorylation with calf intestinal alkaline phosphatase. The data is shown in Fig. 6, Fig. and Fig. , and indicates the presence of a slower migrating, phosphatase-sensitive annexin II form in the nucleus that is not observed in the cytosolic extracts. Thus, phosphorylated annexin II is associated with nuclei in a variety of cells examined.

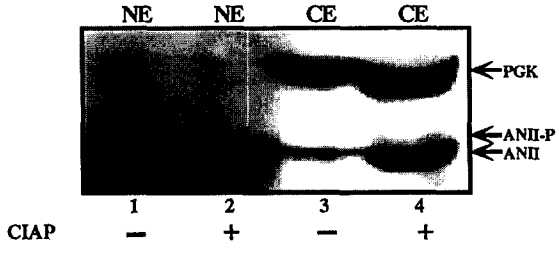

Fig. 6. Phosphorylated annexin II is present in nuclear extracts of K562 cells. Cytosolic (lanes 3 and 4) and nuclear extracts (lanes 1 and 2) from K562 cells were prepared and subjected to treatment with alkaline phosphatase (Lanes 2 and 4). SDS-PAGE and immunoblot analysis with anti-chicken annexin II and anti-PGK antisera was performed. The positions of the PGK, annexin II and phosphorylated annexin II proteins are indicated.

4. Discussion

The physiological role of annexin II remains to be understood. Annexin II is implicated in having a role in a variety of cellular functions. The discovery of annexin II as a major substrate of protein-tyrosine kinases (reviewed in [1]) led to the proposal that annexin II is involved in mitogenic signal transduction. The calcium-dependent phospholipid binding activity along with the plasma membrane association of annexin II heterotetramer may be important for the membrane fusion activity of annexin II. Recent studies on the extracellular annexin II implicate annexin II in a receptor-like function that binds various

molecules such as the tissue plasminogen activator [10,11], cytomegalovirus [23], an alternately spliced form of tenascin-C [24], and as a divalent cation-dependent adhesion molecule [5].

A role for annexin II in cell proliferation and DNA replication is indicated from a number of observations made in the past few years. Annexin II is a growth-regulated gene [25,26] and its expression is cell cycle regulated in mammalian cells [27]. In a complex with phosphoglycerate kinase, called the primer recognition protein complex, annexin II stimulates DNA polymerase α activity [7]. Exposure of HeLa cells to antisense annexin II oligonucleotides reduces the ongoing DNA synthesis [12], and immunodepletion of annexin II results in inability of *Xenopus* egg extracts to replicate exogenous DNA [13]. Overexpression of annexin II is seen in a number of cancer cells and tissues [14–18], and annexin II is present in all cultured cells. The exact mechanism of annexin II action in DNA synthesis and cell proliferation remains to be determined.

We examined 11 human B-cell lymphoma cell lines for annexin II expression, to investigate whether annexin II overexpression seen in other human cancers is also observed in B-cell lymphomas. We observed a wide variation in annexin II expression in these cell lines. While 3 cell lines had unaltered expression when compared to normal B-cells, five cell lines had significant overexpression. Most interestingly, 3 cell lines had significantly reduced or no expression of annexin II. Upon further investigation, we determined that the reduced annexin II expression in these cell lines was regulated at the transcriptional level. Using a sensitive ribonuclease protection assay, we observed a low level of annexin II transcripts in these cell lines. The transcriptional regulation of annexin II in B-cell lymphoma cell lines is consistent with previous observations of annexin II regulation seen in virus-transformed rat cell lines [28], human leukemias [29], and human hepatocarcinoma cells [30]. This transcriptional regulation is not due to methylation of the annexin II gene, since we did not observe reexpression of annexin II upon treatment of cells with demethylating agents.

When immunoblot analyses were conducted with the anti-human recombinant annexin II serum (α774), we did not observe any annexin II protein in Raji and OMA-BL-1 cells. However, when we used the anti-chicken annexin II antiserum, we observed a slower migrating immunoreactive protein. The anti-chicken annexin II antiserum is monospecific for annexin II, and in whole cell extracts of many human and rodent cell lines, we do not see cross-reactivity of this antiserum with any other cellular protein [4,7,12,16]. Thus, Raji and OMA-BL-1 cells produce an altered form of annexin II. Annexin II is an excellent substrate for protein-tyrosine kinases, and we had previously demonstrated the presence of an altered form of annexin II associated with nuclear matrix in HeLa cells [4]. We investigated whether the slower migrating annexin II in Raji and OMA-BL-1 cells is phosphorylated. Dephosphorylation of cell extracts with calf intestinal alkaline phosphatase resulted in a change in the mobility of the altered form. The dephosphorylated protein migrated to the position of the major annexin II protein seen in other cells. Thus, Raji and OMA-BL-1 cells produce a phosphorylated form of annexin II exclusively. The expression of phosphorylated annexin II is not limited to these two cell lines, as shown by the presence of the phosphorylated annexin II in nuclear extracts of K562 cells (Fig. 6) and in HeLa cells [4]. We have observed the slower migrating annexin II in all cell extracts, albeit at a considerably lower level than unphosphorylated annexin II. In the presence of a large excess of unphosphorylated annexin II, it is frequently difficult to observe the phosphorylated form.

The data presented in this paper indicate that cultured cells can survive in the absence of a majority of annexin II, but the phosphorylated form of annexin II may be necessary for cell survival and proliferation. Our results provide the basis for future work on defining the physiological role of annexin II. The role of phosphorylated annexin II in DNA synthesis and cell proliferation needs to be established in future experiments.

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